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Electrophysiological responses of male and female *Amyelois transitella* antennae to pistachio and almond host plant volatiles

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Abstract

The polyphagous navel orangeworm, *Amyelois transitella* Walker (Lepidoptera: Pyralidae), is a major insect pest of almonds and pistachios in California (USA). *Amyelois transitella* moths have proven difficult to monitor and control for over 5 decades; however, recent reports indicate progress towards attractants using pheromone or semiochemical blends. Despite advances of a host plant volatile attractant blend that is effective for monitoring moth populations in almond orchards, the blend's attractancy and capture efficacy of *A. transitella* has not translated to pistachio orchards. The apparent orchard specificity of *A. transitella* to the blend suggests a different composition of host plant volatiles is needed to either improve the current blend or a new blend formulation is required for monitoring in pistachio orchards. One objective of this study was to evaluate available individual host volatiles via a standardised puff method in combination with electroantennographic analysis. In total 105 volatiles were evaluated individually for their ability to elicit an electrophysiological chemoreception response from excised male and female *A. transitella* antennae. Male antennae responded significantly higher to alcohols, aldehydes, alkyls, aromatics, and ketones. Female antennae responded significantly higher to benzenoids, monoterpenes, sesquiterpenes, and short-chain alcohols.

Introduction

The navel orangeworm, *Amyelois transitella* Walker (Lepidoptera: Pyralidae), has been a key insect pest of tree nuts in California (USA) since the 1950s and 1960s (Ebeling, 1959; Michelbacher & Davis, 1961; Wade, 1961; Beck & Higbee, 2013) and continues to inflict significant damage and economic losses to the almond and pistachio industries (Higbee & Siegel, 2009; Niederholzer, 2012). The larvae of navel orangeworm have been shown to vector aflatoxigenic aspergilli fungal spores (Palumbo et al., 2008, 2014) resulting in food safety concerns worldwide regarding mycotoxin contamination of tree nuts (Campbell et al., 2003). Despite numerous efforts over the years, effective control of the navel orangeworm to date has relied primarily on insecticide sprays, proper orchard sanitation (Higbee & Siegel, 2009), and developing use of mating disruption (Higbee & Burks, 2008; Niederholzer, 2012; UC IPM, 2013).

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Numerous efforts to control or monitor *A. transitella* over the years have utilised both pheromonal and non-pheromonal tactics (Beck & Higbee, 2013). The female-produced sex pheromone blend is known (Coffelt et al., 1979; Leal et al., 2005; Kuenen et al., 2010) and a four-component synthetic blend has recently demonstrated good trapping efficacy of male *A. transitella* in tree nut orchards (Beck & Higbee, 2013).

Host plant volatiles have been shown to play a large role in attracting herbivorous insects (Bruce et al., 2005; Norin, 2007; Bruce & Pickett, 2011; Pickett et al., 2012; Najar-Rodriguez et al., 2013). Generalist (i.e., polyphagous) insects or multivoltine insects with several generations within a growing season are likely to encounter volatile bouquets with phenologically progressive or complex chemical compositions (Tasin et al., 2005; Bruce & Pickett, 2011; Braasch et al., 2012; Najar-Rodriguez et al., 2013). As such, phytophagous insects such as the navel orangeworm are thought to recognise chemical cues through varying mechanisms, such as specific ratios of ubiquitous host plant volatiles (Bruce et al., 2005) or background odours enhancing orchard-specific volatiles

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(Schröder & Hilker, 2008). Thus, it is important to consider the individual odourants of each crop and their phenological expression when evaluating the host plant volatiles for semiochemical activity.

A synthetic blend of host plant volatiles has been recently shown to attract both sexes of navel orangeworm in almond orchards (Beck et al., 2012a); however, this blend, which was based on almond tissue emissions, did not efficaciously attract navel orangeworm in pistachio orchards (Beck & Higbee, 2013; Beck et al., in press). Subsequently, our laboratories have undertaken the task of developing a blend of volatiles attractive to *A. transitella* in pistachio orchards. Using identified and available volatiles, and a standardised method for each volatile (Beck et al., 2012b), we have employed electroantennographic (EAG) analysis as a means to screen the numerous volatiles from both almond and pistachio tissue matrices for their chemoreception specificity by female and male *A. transitella*.

There are various approaches for the development of blends of varying chemical composition, including demarcation by chemical class, EAG response amplitude, or concentrations based on relative amounts (Jang et al., 1989; Park et al., 2002; Beck et al., 2012b). In addition, the varying responses of female and male antennae to structural classes of compounds (Raguso et al., 1996) should be considered. Due to the high number of volatiles included herein the objective of this report was to make available the extensive EAG results and thus provide data for different approaches for the development of new blends based on the chemoreceptivity of *A. transitella*.

Materials and methods

Gas chromatography-mass spectrometry (GC-MS) analysis

All volatiles tested were checked by GC-MS for purity and verification of identities. The volatiles' retention indices were compared with an internal database and the volatile identity further confirmed using NIST (NIST02) and Wiley (7th) fragmentation pattern databases. Volatiles were analysed on either a J&W Scientific (Folsom, CA, USA) DB-Wax column or a J&W Scientific DB-1 (both 60 m × 0.32 mm inner diameter × 0.25 μm), installed on HP-6890 GCs coupled to HP-5973 mass selective detectors (Palo Alto, CA, USA). The instrument parameters and methods used were identical to those previously published (Beck et al., 2009).

Volatile preparation for bioassay

Solutions of each volatile were prepared at a concentration of 5 mg ml^{-1} in pentane (VWR, Radnor, PA, USA), transferred to a glass vial, tightly sealed, and either immediately used or refrigerated until used. If refrigerated, the sealed vial was allowed to warm to room temperature prior to use. The same procedure previously described (Beck et al., 2012b) was used for the loading of the material onto oven-dried 6.0-mm-diameter assay discs (Whatman; Sigma-Aldrich, St. Louis, MO, USA) and insertion into Pasteur pipets (VWR) for odour/puff delivery. Briefly, a 50-μl glass syringe was used to deliver 10 μl of the 5 mg ml^{-1} solution onto the assay disc, the pentane solvent was allowed to evaporate from the disc for 2 min, the disc inserted into a labelled, disposable 14.3-cm-long Pasteur pipet, and the ends sealed with Parafilm (VWR).

Electroantennographic (EAG) analysis

Insect rearing and electrophysiological recording protocols used were identical to previously published (Beck et al., 2012b). Briefly, individual 3- to 4-day-old male and female moths were transferred into small, lidded plastic containers the morning of the assay. Moths were assumed to have mated as they were allowed to cohabitate in the same jar from the time they emerged as adults until their removal from the jar just prior to evaluation. Approximately 12 min before each experiment, the moth to be tested was transferred head first into a holding apparatus (i.e., made from various plastic pipettor tips) and secured from behind. Manipulation of the antennae was viewed under a low-power stereo-microscope to facilitate excision. The moth antennae were teased out using a wire-tipped tool. The electrode fork holder (Syntech, Kirchzarten, Germany) with a small bead of electrode gel (Parke, Fairfield, NJ, USA) was placed in close proximity for quick transfer of the excised antennae. The fork holder with the excised antennae was immediately connected to the pre-amplifier and placed under a stream of humidified air (200 ml per min). Antennal responses to the individual volatiles were started exactly 10 min after antennae excision and recorded on a 4-channel acquisition controller (Syntech, Hilversum, The Netherlands). Each puff stimulation diverted the airstream for a 2-s duration through the test volatile pipet and onto the antennal preparation with a 1-min recovery period between puffs of volatiles. This study spans a 3-year series of experiments conducted to screen the 105 test volatiles. Each excised antennae pair was exposed to sets of 4–6 individual test volatiles for both female and male antennae. In addition, puffs of acetophenone as a positive control/standard (Beck et al., 2012b) were delivered at the beginning and end of each experiment. Pentane (10 μl) prepared under identical conditions was used as a negative control and puffed during the midpoint of the treatments for each experiment. For all replicate sample puffs, the order of volatiles presented was randomised.

Data analysis

For each EAG experiment, the antennal response amplitude in μV to the negative control was subtracted from the antennal response to each individual volatile, including the control/standard acetophenone. Next, the antennal responses of the two acetophenone puffs (first and last puffs of each experiment), minus the negative control response, were averaged and then corrected to a value of 1 000 μV for the positive control (Beck et al., 2012b). Raw EAG responses of the female and male antennae to acetophenone (mean ± SEM; n = 50 each) were 1 137 ± 30 and 1 262 ± 35 μV, respectively (t-test: $t = -2.968$, d.f. = 98, $P = 0.008$). EAG response amplitudes to the test volatile puffs were converted to the proportion of the average corrected response to acetophenone within each set. The corrected replicate values for each experiment were then pooled, averaged, and reported. Statistical analyses were performed using SigmaStat, version 4.0 (Systat Software, San José, CA, USA). Normality of the EAG μV data was analysed with the Shapiro–Wilks test. If response data were not normal in distribution then they were $\sqrt{(x + 0.05)}$-transformed prior to analysis of variance (ANOVA). Normalised data for each class of volatiles were analysed with ANOVA, and if significant effects were found the means were separated by Tukey's test. For each volatile, female and male EAG μV response data were compared using a t-test.

Results and discussion

In total 105 volatiles from almond and pistachio matrices (Tables 1 and 2) were available for the survey of electrophysiological responses of female and male navel orangeworm antennae. Of the 105 available volatiles, 55 were associated with almond emissions, 29 with pistachio emissions, and 21 were associated with both orchards (Beck et al., 2008, 2009, 2011a,b, 2012a; Roitman et al., 2011; Mahoney et al., 2014). The relatively low number of available pistachio volatiles for this study is not fully representative of pistachio volatile profiles, as pistachios in general emit a larger number of volatiles, typically terpenoids, than almonds (Beck et al., 2009, 2014b; Roitman et al., 2011). For purposes of analysis and discussion, the 105 volatiles were delineated into 12 broad structural classes of compounds (Figure 1) – alkyl (4), short-chain alcohols (6), alcohols (5), spiroketals (2), aromatics (2), benzenoids (18), aldehydes (6), ketones (7), esters (10) with one further classified as an acid, lactones (3), monoterpenes (22), and sesquiterpenes (20).

For all classes of volatiles analysed in Table 1 the female antennae had an average response of 532 μV (range 17–1 885 μV). Similarly, male antennae had an average response of 573 μV (range 66–1 754 μV). Largely, the female and male pooled antennal responses were statistically different between the sexes, with female responses greater for the classes representing short-chain alcohols, benzenoids, monoterpenes, and sequiterpenes (Figure 1). The male responses were greater for the classes of alkyls, aromatics, and ketones, and dominantly larger for the alcohol and aldehyde classes. This increased response by male antennae to the aldehyde moiety has been noted by Liu et al. (2010) regarding components of the female-produced sex pheromone bouquet, which contains several long-chain aldehyde components.

Sexual dimorphic differences were observed for 55% of the test volatiles with antennal responses significantly greater for 24 volatiles for females and 34 volatiles for males (Table 1). When the female and male antennal responses to individual volatiles were compared, the electrophysiological differences between classes were further delineated. The average female antennal response to the top 25 volatiles, eliciting the highest responses from the navel orangeworm, was 1 034 μV (range 732–1 885 μV) (Figure 2). Surprisingly, the female antennae responded more favourably to monoterpenes, with nine of the top 25 volatiles in that class, and seven of the monoterpenes in the top 10 volatiles. Notably, three of the seven monoterpenes in the top 10 possessed an alcohol moiety. The nine monoterpenoids in the top 25 volatiles for females include the most stimulating volatile, sabinene hydrate, followed by (*Z*)-ocimene, (S)-α-pinene, terpine-4-ol, linalool, α-terpinolene, Δ^3-carene, (R)-limonene, and (*E*)-β-ocimene.

There are reports of monoterpenes serving as either ovipositional or host-locating attractants for other lepidopterans (Städler, 1974; Fatzinger & Merkel, 1985; Leather, 1987; Shu et al., 1997). More specifically, monoterpenes such as α-pinene, β-pinene, limonene, and myrcene have been shown to elicit ovipositional behaviour from other pyralid moths (Fatzinger & Merkel, 1985; Shu et al., 1997). α-Pinene and limonene were listed in the top 25 volatiles for eliciting high EAG responses from the female navel orangeworm antennae, but researchers have noted discrepancies or poor correlations between the degree of actual behavioral activity and electrophysiological response amplitudes of other Pyralidae and certain monoterpenes (Shu et al., 1997). For instance, antennae of the fir coneworm moth, *Dioryctria abietivorella* Grote, responded with low amplitude EAG responses to limonene and higher amplitude responses to both myrcene and Δ^3-carene, whereas limonene stimulated a moderate and the other two a stronger ovipositional response in behavioural assays (Shu et al., 1997). To exemplify electrophysiological differences among pyralid species, where the fir coneworm moth responded well in EAG assays to

Table 1 Electroantennographic responses (μV) from female and male navel orangeworm moths to volatiles (50 μg) detected from various almond and pistachio matrices. EAG values are corrected to antennal responses of 1 000 μV to acetophenone

			♀ EAG[3]		♂ EAG[3]		♀ vs. ♂ t-test[4]					
Class[1]	RI[2]	Compound identity	Mean ± SE (μV)	Rank	Mean ± SE (μV)	Rank	t	P	Crop[5]	Orig[6]	% GC[7]	Reference[8]
Alkyl	1306	(*E*)-4,8-Dimethyl-1,3,7-nonatriene	402 ± 38	58	665 ± 38b	36	−4.901	<0.001	A,P	b	55	1,5,10,12
	1039	1-Decene	499 ± 62	43	713 ± 74b	33	−2.222	0.043	A	a	95	12
	1099	Undecane	379 ± 106	63	958 ± 108a,b	20	−3.832	0.005	A	c	99	3,4
	1243	1-Dodecene	612 ± 52	32	1014 ± 94a	16	−3.729	0.003	A	a	97	1,4
SCA	1019	2-Butanol, [R]-	271 ± 21c,d	84	136 ± 38d	100	2.895	0.018	A	d	99	1
	1020	2-Butanol, [S]-	176 ± 33d	97	164 ± 15d	93	0.330	0.75	A	d	100	1
	1208	2-Methyl-1-butanol	790 ± 56a,b	24	419 ± 26c,d	54	6.053	<0.001	A	e	99	1
	1209	3-Methyl-1-butanol	576 ± 43b,c	36	259 ± 32d	76	5.942	<0.001	A	a	99	1,8
	1249	1-Pentanol	183 ± 25d	96	369 ± 43c,d	64	−3.594	0.003	A	a	99	8
	1107	3-Pentanol	486 ± 80b,c,d	46	376 ± 28c,d	63	1.212	0.25	A	f	94	1
Alcohol	1353	1-Hexanol	584 ± 55b,c	33	1658 ± 133a	2	−7.456	<0.001	A	a	99	4,6–8
	1384	(*Z*)-3-Hexen-1-ol	1014 ± 173a	12	803 ± 95b	27	1.068	0.31	A,P	g	100	2,6,7,10
	1321	2-Heptanol	849 ± 90a,b	18	1700 ± 84a	1	−6.877	<0.001	A	a	92	8
	1451	1-Octen-3-ol (racemic)	1070 ± 155a	9	1526 ± 144a	5	−2.141	0.050	A	h	98	1,5,8
	1722	Undecan-2-ol	268 ± 40c,d	86	610 ± 34b,c	38	−6.551	<0.001	A	d	97	2
Spiroketal	1348	(+/-)-Chalcogran (*E,Z* mix)	697 ± 50	26	992 ± 56a	19	−3.581	0.004	A	i	93	1,6–8
	1286	(+/-)-(*E*)-Conophthorin	732 ± 14	25	676 ± 72b	35	0.759	0.46	A	i	98	1,5–8
Aromatic	2428	1*H*-Indole	198 ± 36	94	158 ± 18b	94	1.003	0.35	P	f	98	10
	1228	2-Pentylfuran	321 ± 44	76	607 ± 52a	40	−4.021	0.001	A	h	98	1,4,7–9
Benzenoid	1169	Cumene	559 ± 128c,d,e	39	408 ± 66d,e,f	59	1.239	0.24	A	f	93	3
	1252	Styrene	206 ± 28e	92	189 ± 47f	86	0.336	0.74	A	a	99	1,2,9
	1598	Benzonitrile	358 ± 32d,e	67	342 ± 44e,f	66	0.454	0.66	A	b	100	3
	1999	Phenol	338 ± 55d,e	73	445 ± 59d,e,f	49	1.243	0.25	A	a	99	3–5,12
	1906	2-Phenylethanol	462 ± 30d,e	50	1084 ± 112a,b	14	−5.726	<0.001	A	a	93	1,2,5,8
	1635	Phenylacetaldehyde	566 ± 38b,c,d,e	37	400 ± 39d,e,f	56	2.578	0.026	A	a	96	3
	1515	Benzaldehyde	491 ± 101d,e	45	410 ± 54d,e,f	55	0.709	0.50	A	f	99	1,3,5,8,9
	1644	Acetophenone	1000a	13	1000a	18	–	–	A	h	100	3,5
	2018	*p*-Anisaldehyde	336 ± 72d,e	75	554 ± 78d,e	44	−1.735	0.12	A	j	97	3
	1668	Salicylaldehyde	1246 ± 85a	4	594 ± 60c,d,e	41	6.047	<0.001	A	a	100	3
	1616	Methyl benzoate	806 ± 70a,b,c,d	21	734 ± 60b,c,d	31	0.740	0.48	A,P	m	100	3,5,8,10,12
	1661	Ethyl benzoate	1102 ± 180a,b	8	499 ± 91d,e,f	45	2.653	0.022	A	h	99	1,3,5,8
	2119	(*Z*)-3-Hexenyl benzoate	617 ± 34c,d,e	31	872 ± 54b,c	23	−4.085	0.001	P	k	97	10

Table 1. Continued

Class[1]	RI[2]	Compound identity	♀ EAG[3] Mean ± SE (μV)	Rank	♂ EAG[3] Mean ± SE (μV)	Rank	♀ vs. ♂ t-test[4] t	P	Crop[5]	Orig[6]	% GC[7]	Reference[8]
	1769	Methyl salicylate	1036 ± 281a,b,c	11	430 ± 80d,e,f	53	1.934	0.079	A,P	h	98	3,5,10,12
	2225	Methyl anthranilate	269 ± 28e	85	192 ± 49f	85	1.365	0.19	A	l	96	2
	2537	Vanillin	400 ± 37d,e	60	474 ± 51d,e,f	46	−1.170	0.26	A	f	98	1
	1733	Naphthalene	459 ± 109d,e	52	326 ± 30e,f	68	1.418	0.19	A	n	99	3
	1878	1-Methylnaphthalene	282 ± 79e	81	243 ± 79e,f	80	0.353	0.73	A	f	67	3
Aldehyde	1077	Hexanal	360 ± 71b	71	719 ± 74c	32	−3.587	0.005	A	h	97	3,4,7,12
	1182	Heptanal	467 ± 62a,b	49	1140 ± 132a,b,c	11	−4.197	<0.001	A	a	98	3,4
	1286	Octanal	428 ± 94a,b	54	1108 ± 114a,b,c	13	−4.624	<0.001	A	h	93	3,4
	1390	Nonanal	554 ± 98a,b	40	1451 ± 113a	7	−5.787	<0.001	A	a	99	a,b,c
	1532	Non-2-enal	683 ± 39a	27	1031 ± 68b,c	15	−4.419	<0.001	A	b	53	1–4,7–9
	1495	Decanal	418 ± 91a,b	55	1298 ± 100a,b	10	−6.333	<0.001	A	a	96	3,4,8,12
Ketone	1279	3-Hydroxy-2-butanone	85 ± 20d	103	139 ± 45d	99	−1.086	0.29	A	a	51	1,5,8
	1177	2-Heptanone	344 ± 34b	44	690 ± 73c	34	−2.776	0.020	A	a	99	4,8
	1283	2-Octanone	459 ± 67b,c	51	654 ± 32c	37	−2.311	0.043	A	a	98	4,8
	1251	3-Octanone	816 ± 85a	20	1007 ± 82b	17	−1.622	0.13	A	a	99	1,8
	1403	3-Octen-2-one	803 ± 74a	22	1588 ± 83a	4	−7.045	<0.001	A	g	100	4
	1598	2-Undecanone	484 ± 56b,c	47	1135 ± 66b	12	−7.611	<0.001	A,P	o	97	2,10
	1852	Geranylacetone	231 ± 40c,d	90	233 ± 21d	82	−0.039	0.97	A	d	97	2
Ester	1047	Ethyl 2-methylbutyrate	434 ± 48c,d	53	328 ± 20c	67	2.031	0.059	A	b	76	1,8
	1063	Ethyl 3-methylbutyrate	257 ± 31d	87	208 ± 29c	84	1.138	0.28	A	b	97	1,8
	1029	Ethyl butyrate	316 ± 38d	78	236 ± 35c	81	1.547	0.15	A	f	99	1
	1232	Ethyl hexanoate	875 ± 81a	17	861 ± 58b	24	0.140	0.89	A	h	98	1,8
	1271	Hexyl acetate	550 ± 45b,c	41	768 ± 60b	30	−2.891	0.010	A	a	99	2,7
	1315	(*Z*)-3-Hexenyl acetate	651 ± 72a,b,c	29	922 ± 51b	22	−2.763	0.016	A,P	g	97	2,5,7,10
	1460	(*Z*)-3-Hexenyl butyrate	802 ± 60a,b	23	808 ± 81b	26	−0.060	0.95	A,P	p	100	2,10,12
	1679	Decyl acetate	561 ± 53b,c	38	1359 ± 136a	9	−6.259	<0.001	A	b	86	2
	2247	Ethyl palmitate	400 ± 34c,d	59	410 ± 46c	58	0.064	0.95	A*	f	63	12
Acid	1443	Acetic acid	239 ± 31	88	134 ± 25	101	2.633	0.022	A	n	99	3,4,8,9
Lactone	1619	γ-Butyrolactone	162 ± 23b	98	181 ± 21b	89	−0.564	0.59	A	j	87	2,4,8,9
	1602	γ-Pentanolactone	283 ± 82b	80	357 ± 35b	65	−0.962	0.36	A	a	96	3,9
	1694	γ-Hexanolactone	645 ± 136a	30	791 ± 101a	28	−0.848	0.42	A	b	97	3,4,9
Monoterpene acyclic	1232	(*Z*)-Ocimene	1605 ± 196a,b	2	444 ± 84d,e,f	50	6.074	<0.001	P	p	63	9,10
	1248	(*E*)-β-Ocimene	848 ± 54d,e,f	19	934 ± 58b	21	−1.092	0.30	P	b	93	5,9,10,12

Table 1. Continued

Class[1]	RI[2]	Compound identity	♀ EAG[3]		♂ EAG[3]		♀ vs. ♂ t-test[4]		Crop[5]	Orig[6]	% GC[7]	Reference[8]
			Mean ± SE (μV)	Rank	Mean ± SE (μV)	Rank	t	P				
	1548	Linalool	1202 ± 47b,c,d	6	1453 ± 55a	6	−3.514	0.003	A,P	m	95	2,9,10
	1158	β-Myrcene	128 ± 46i,j	100	254 ± 34e,f,g	77	−2.201	0.048	A,P	p	88	1,5,9–12
Monocyclic	1265	*p*-Cymene	413 ± 86f,g,h,i,j	56	471 ± 68c,d,e,f	48	−0.535	0.61	A,P	f	99	1,3,9,10,12
	1161	α-Phellandrene	580 ± 62e,f,g,h	34	398 ± 50d,e,f,g	57	2.267	0.043	P	b	60	10
	1176	α-Terpinene	352 ± 82g,h,i,j	69	390 ± 54d,e,f,g	61	−0.394	0.70	P	a	82	9,10,12
	1242	γ-Terpinene	362 ± 34g,h,i,j	64	212 ± 30e,f,g	83	3.328	0.003	P	a	92	8–10,12
	1279	α-Terpinolene	1122 ± 72c,d	7	574 ± 48c,d	42	6.406	<0.001	P	p	90	8–12
	1601	Terpineol-4	1213 ± 86b,c,d	5	1364 ± 104a	8	−1.119	0.29	P	a	97	10,12
	1693	α-Terpinyl acetate	470 ± 80f,g,h,i	48	186 ± 36f,g	87	3.228	0.005	P	s	57	10
	1197	Limonene, [R]-	903 ± 92c,d,e	14	569 ± 112c,d	43	2.320	0.039	A,P	a	97	1,3–5,8–12
	1197	Limonene, [S]-	662 ± 84e,f	28	430 ± 18d,e	51	2.684	0.014	A,P	a	96	1,3–5,8–12
Bicyclic	1018	α-Pinene, [1R]-	580 ± 100e,f,g,h	35	378 ± 48d,e,f	62	1.825	0.085	A,P	a	99	1,5,8–12
	1018	α-Pinene, [1S]-	1297 ± 116b,c	3	267 ± 28e,f,g	75	10.252	<0.001	A,P	h	99	1,5,8–12
	1105	β-Pinene	320 ± 24g,h,i,j	77	274 ± 60e,f,g	74	0.712	0.49	A,P	k	95	1,5,9–11
	1062	Camphene	17 ± 17j	105	323 ± 37d,e,f,g	69	−7.643	<0.001	A,P	b	86	1,5,9–12
	1022	α-Thujene (racemic)	390 ± 30g,h,i,j	62	174 ± 22f,g	91	5.603	<0.001	P	r	80	8–12
	1144	Δ^3-Carene	1052 ± 44c,d	10	776 ± 42b,c	29	4.504	<0.001	P	b	92	9–12
	1117	Sabinene (racemic)	362 ± 106g,h,i,j	66	156 ± 54f,g	96	1.739	0.12	P	r	81	9,10
	1468	Sabinene hydrate	1885 ± 114a	1	1598 ± 98a	3	1.908	0.081	P	d	93	12
	1577	Bornyl acetate	232 ± 62h,i,j	89	74 ± 8g	104	2.527	0.035	P	q	95	8–10
Sesquiterpene acyclic	1356	Farnesane	339 ± 27c,d,e	72	253 ± 30d,e,f	78	2.155	0.047	A	a	72	2
	1747	(*E*,*E*)-α-Farnesene[9]	408 ± 50b,c,d	57	430 ± 30b,c,d	52	−0.359	0.73	P	t	71	5,10,12
	1664	(*E*)-β-Farnesene	400 ± 80b,c,d	61	472 ± 32b,c	47	−0.834	0.42	P	g	95	9,10
	2040	Nerolidol	278 ± 28d,e,f	83	392 ± 28c,d	60	−2.721	0.020	P	b	38	12
Monocyclic	1705	Germacrene-D	120 ± 43d,e,f	101	323 ± 35c,d,e	70	−3.707	0.006	A,P	b	96	2,9,10
	1665	α-Humulene	548 ± 29b,c	42	279 ± 11c	73	7.481	<0.001	A,P	a	97	2,5,10
Bicyclic	1817	Cuparene	100 ± 38e,f	102	154 ± 30e,f,g	97	−1.136	0.28	P	b	92	9,12
	1715	Valencene	879 ± 48a	16	856 ± 48a	25	0.318	0.76	P	g	83	10
	1759	γ-Cadinene	196 ± 46d,e,f	95	66 ± 6g	105	2.430	0.025	P	b	66	10
	1717	β-Selinene	898 ± 70a	15	608 ± 72b	39	2.889	0.009	P	b	61	10
	1594	β-Caryophyllene	502 ± 36c,d,e	70	360 ± 36e,f,g	92	3.889	<0.001	A,P	b	87	2,5,10

Table 1. Continued

Class[1]	RI[2]	Compound identity	♀ EAG[3]		♂ EAG[3]		♀ vs. ♂ t-test[4]		Crop[5]	Orig[6]	% GC[7]	Reference[8]
			Mean ± SE (μV)	Rank	Mean ± SE (μV)	Rank	t	P				
Tricyclic	1605	Aromadendrene	222 ± 42d,e,f	91	118 ± 22e,f,g	102	2.097	0.060	A,P	d	99	1,10
	1644	Alloaromadendrene	146 ± 20d,e,f	99	138 ± 16e,f,g	98	0.268	0.79	P	d	98	10
	1456	α-Cubebene	296 ± 50d,e,f	79	252 ± 38d,e,f	79	0.719	0.48	P	b	97	10
	1538	β-Cubebene	198 ± 20d,e,f	93	94 ± 26f,g	103	3.220	0.005	P	b	77	10
	1618	Thujopsene	336 ± 66c,d,e,f	74	178 ± 26e,f,g	90	2.687	0.02	P	u	93	9,12
	1481	α-Ylangene	356 ± 46b,c,d	68	310 ± 36c,d,e	72	0.770	0.45	P	v	90	9,10
	1490	α-Copaene	361 ± 22b,c,d,e	65	315 ± 17c,d,e	71	1.691	0.12	A,P	b	86	2,10
	1693	Ledene	280 ± 14d,e,f	82	184 ± 30e,f,g	88	3.131	0.008	P	d	93	10
	1464	α-Longipinene	70 ± 30f	104	156 ± 22e,f,g	95	−2.377	0.039	P	d	96	9,12

[1]Compound class by major functional group or class. SCA, short-chain alcohol (fewer than six carbon atoms).

[2]Retention indices based on calculated values of authentication standards, relative to n-alkanes on a DB-wax column.

[3]Means within a class of volatiles and within a column followed by a different letter are significantly different (Tukey's test: $P<0.05$).

[4]Comparison of female and male EAG responses to each volatile (t-test).

[5]Volatiles detected from: A, almond matrices; P, pistachio matrices.

[6]Source of chemicals used for EAG analyses: a, Sigma-Aldrich; b, isolated by previous ARS scientist (see also Jang et al., 1989); c, Poly Science; d, Fluka; e, Chem Sample; f, Eastman; g, Bedoukian; h, Alfa Aesar; i, Contech; j, Chem Supply; k, TCI America; l, ChemBo Pharma; m, Fritzsche; n, Fisher; o, Agros; p, SAFC; q, Givaudan-Delawanna; r, Haarman & Reimer; s, Spectrum Chemical; t, Suterra; u, Freeman; v, Hirose.

[7]Purity (%) by headspace solid phase microextraction gas chromatography-mass spectroscopy analysis.

[8]Reference for report of volatile detection: 1, Beck et al. (2008); 2, Beck et al. (2009); 3, Beck et al. (2011a); 4, Beck et al. (2011b); 5, Beck et al. (2012a); 6, Beck et al. (2012c); 7, Mahoney et al. (2014); 8, Beck et al. (2014b); 9, Beck et al. (2014a); 10, Roitman et al. (2011); 11, Dragull et al. (2010); 12, unpublished results.

[9]Purity (%) value reported for this compound was performed by injection by Suterra, the company that synthesised the compound for this and other relevant studies.

Table 2 Statistical values (ANOVA or t-test) for comparison of compounds within a class for female and male antennal responses

	F/t and P values	
Class[1]	♀	♂
Alkyl	$F_{3,25} = 2.908$, $P = 0.054$	$F_{3,25} = 5.469$, $P = 0.005$
SCA/Alcohol	$F_{10,69} = 15.088$, $P<0.001$	$F_{10,72} = 59.081$, $P<0.001$
Spiroketal	$t_{12} = 0.664$, $P = 0.52$	$t_{12} = 3.170$, $P = 0.008$
Aromatic	$t_{12} = 2.032$, $P = 0.070$	$t_{12} = 6.165$, $P<0.001$
Benzenoid	$F_{17,142} = 20.502$, $P<0.001$	$F_{17,150} = 43.340$, $P<0.001$
Aldehyde	$F_{5,38} = 2.768$, $P = 0.032$	$F_{5,40} = 4.419$, $P = 0.003$
Ketone	$F_{6,44} = 23.638$, $P<0.001$	$F_{6,39} = 68.169$, $P<0.001$
Ester	$F_{8,60} = 12.691$, $P<0.001$	$F_{8,54} = 38.176$, $P<0.001$
Lactone	$F_{2,13} = 10.376$, $P = 0.002$	$F_{2,18} = 18.666$, $P<0.001$
Monoterpene	$F_{21,135} = 35.570$, $P<0.001$	$F_{21,145} = 60.803$, $P<0.001$
Sesquiterpene	$F_{19,129} = 24.499$, $P<0.001$	$F_{21,137} = 30.423$, $P<0.001$

[1]Compound class by major functional group or class. SCA, short-chain alcohol (fewer than six carbon atoms).

myrcene, in our study the antennae of both female and male navel orangeworm responded poorly: 128 and 254 μV, respectively.

In addition to several volatiles from this class of compounds eliciting high EAG responses (Figure 2), the importance of monoterpenes for the female antennae is corroborated by two recent studies: Nay et al. (2012) showed that pistachio mummies attracted female navel orangeworm moths to traps in both almond and pistachio orchards, and work in our laboratories confirmed pistachio mummies evoke both attraction and oviposition by female navel orangeworm (Beck et al., in press). The volatile profile of pistachio mummies was determined to comprise primarily monoterpenes, including (*Z*)-ocimene, α-pinene, limonene, and others (Beck et al., 2014a).

Another noteworthy observation from Figure 2 and the top 25 volatiles eliciting female antennal responses was the inclusion of all five components of a synthetic host plant volatile blend shown for the past 3 years to attract both sexes of navel orangeworm moths in almond orchards (Beck et al., 2012a; Beck & Higbee, 2013). Ethyl benzoate (ranking 8th), 1-octen-3-ol (9), methyl salicylate (11), acetophenone (13), and conophthorin (25) all elicited fairly strong responses, with ethyl benzoate, methyl salicylate, and conophthorin eliciting higher responses from the female than the male antennae.

The top 25 volatiles that elicited the highest amplitude responses from male navel orangeworm antennae shared some similarities with the top 25 volatiles for female antennae, but also demonstrated some important differences (Figure 3). The average response for the male was 1 194 μV – a little higher than the female value – and the male top 25 had a range of 857–1 700 μV. Eleven of the 25 volatiles – 2-heptanol, sabinene hydrate, 3-octen-2-one, 1-octen-3-ol, linalool, terpineol-4, 3-octanone, acetophenone, (*E*)-β-ocimene, ethyl hexanoate, and valencene – were common to both lists of the top 25 EAG elicitors. Moreover, where the female top 25 volatiles had all five components of the bisexually attractive synthetic blend, only two of the volatiles, 1-octen-3-ol and acetophenone, were in the top 25 volatiles for males, with the other co-attractant volatiles ranking at 35 (conophthorin), 45 (ethyl benzoate), and 53 (methyl salicylate). In almond orchards this synthetic five-component blend attracts wild male moths in slightly greater numbers than female moths (Beck et al., 2012a), despite the chemoreceptive sensitivities/affinities to these key volatiles being greater for female navel orangeworm antennae.

The most striking difference between the male and female top 25 volatiles was the composition of the classes of compounds evoking the highest male responsiveness. Only four of the 25 volatiles for male sensitivity were monoterpenes (cf. nine for female sensitivity) and three of those were monoterpenoid alcohols. Moreover, for the top 25 stimulatory volatiles for males there were large increases in the number of fatty acid breakdown products (alkanals) and fungal volatiles (alkanols and alkanones) over those in the female ranking of volatiles. This apparent trend of the males' higher response to volatiles that are present in or derived from further stages of nut decay (fungal or oxidative) would need to be further investigated for their semiochemical activity. Several of the volatiles noted in Figure 3 were also detected in wet pistachio and almond mummies (Beck et al., 2014b), as well as from fungal-contaminated almond kernels (Beck et al., 2011b). One possibility for this could be that males are attracted to decayed nuts, such as mummies from which *A. transitella* emerge (Kuenen & Siegel, 2010). It is obligatory for their survival that the non-diapausing navel orangeworm moths are attracted to post-harvest nuts remaining in the orchard primarily as stick-tights and mummies. Navel orangeworm larvae develop and then emerge as adults during the prolonged period from late summer harvest through the development of the next nut crop the following spring. The majority of mating of *A. transitella* moths has been shown to occur

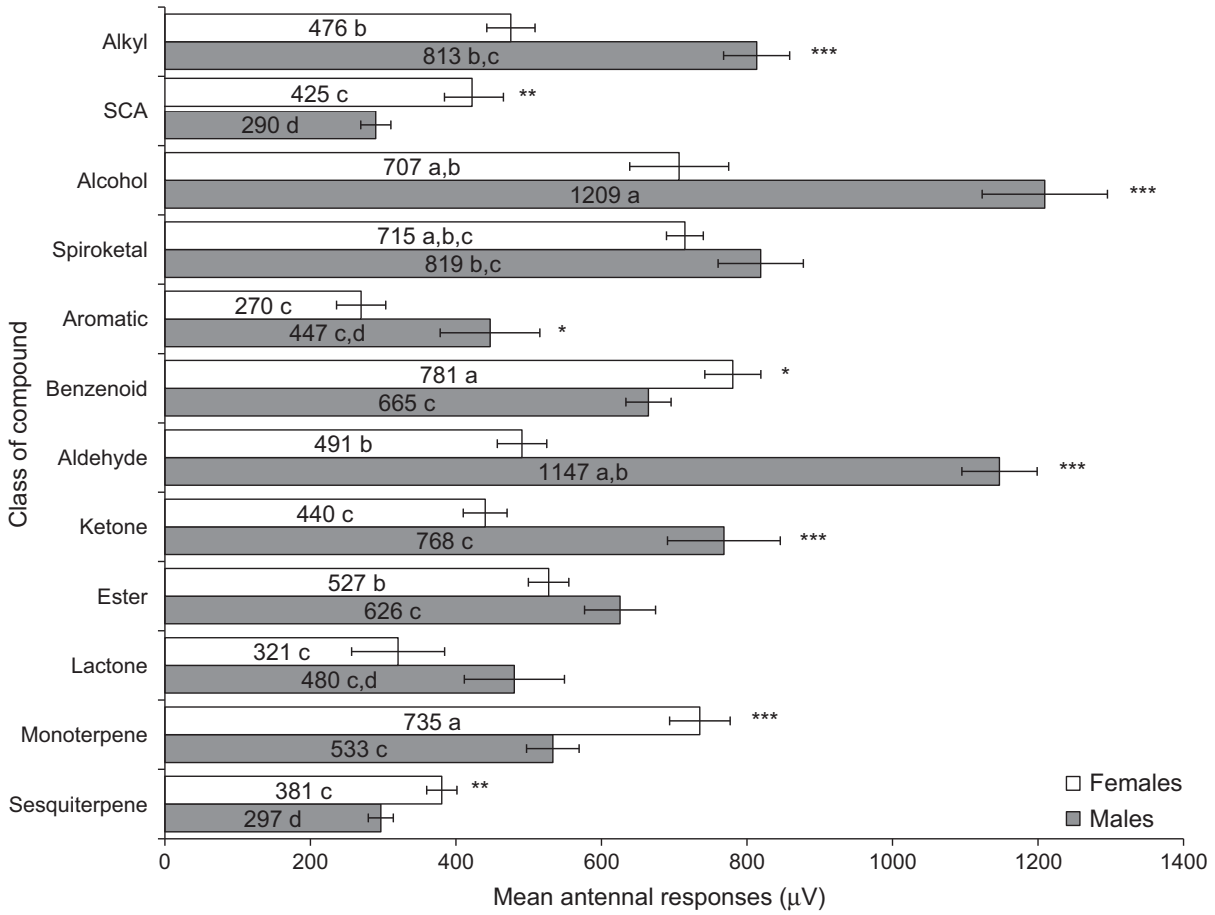

Figure 1 Mean (± SE) electroantennographic responses (μV) of female and male *Amyelois transitella* to the defined classes of compounds (described in Table 1). Values are means of the replicate antennal responses to the compounds within each class. Different letters in the bars denote significant differences between classes within a sex (Tukey's test: $P<0.05$). Significant differences between female and male values for a particular class of compounds are denoted by *$0.01<P<0.05$, **$0.001<P<0.01$, and ***$P<0.001$ (t-test). SCA, short-chain alcohol (fewer than six carbon atoms).

within the first 2 days after emergence (Parra-Pedrazzoli & Leal, 2006). The attraction of male *A. transitella* to mummies might increase their chances of mating with a female as she emerges from mummy nuts during early spring flights.

Also of interest in this study were certain observed structure-activity aspects or subtle changes in chemical structure that appeared to affect the amplitude of EAG responses. These subtle structural changes may suggest evolved higher sensitivities and affinities for particular volatiles over other related volatiles in the various class groupings. A number of structural differences in closely related volatiles had significant effects on the evoked EAG amplitudes, including differences in chain length, functional group, configuration of alkene, and constitutionally isomeric acyclic, cyclic, and polycyclic configurations of the volatiles. For instance, an increase in aliphatic chain length generally appeared to be associated with increasing EAG responsiveness for various classes of compounds. This observation included the alkyls with 1-dodecene eliciting the highest antennal response, short-chain vs. longer chain alcohols, ketones, esters (for males in particular), and the lactones.

Addition of an alkene moiety had varying effects on overall female and male antennal responses. For instance, for male antennae 1-hexanol elicited a significantly higher amplitude response than (*Z*)-3-hexen-1-ol, yet the opposite, and significant response was noted for the female antennae for these two volatiles. A second example was nonanal and non-2-enal, which elicited significantly different responses from male antennae. For the ketones, placement of the carbonyl, or alternatively the length of the side chain may have an effect on antennal responses. For instance, 2-octanone and 3-octanone elicited significantly

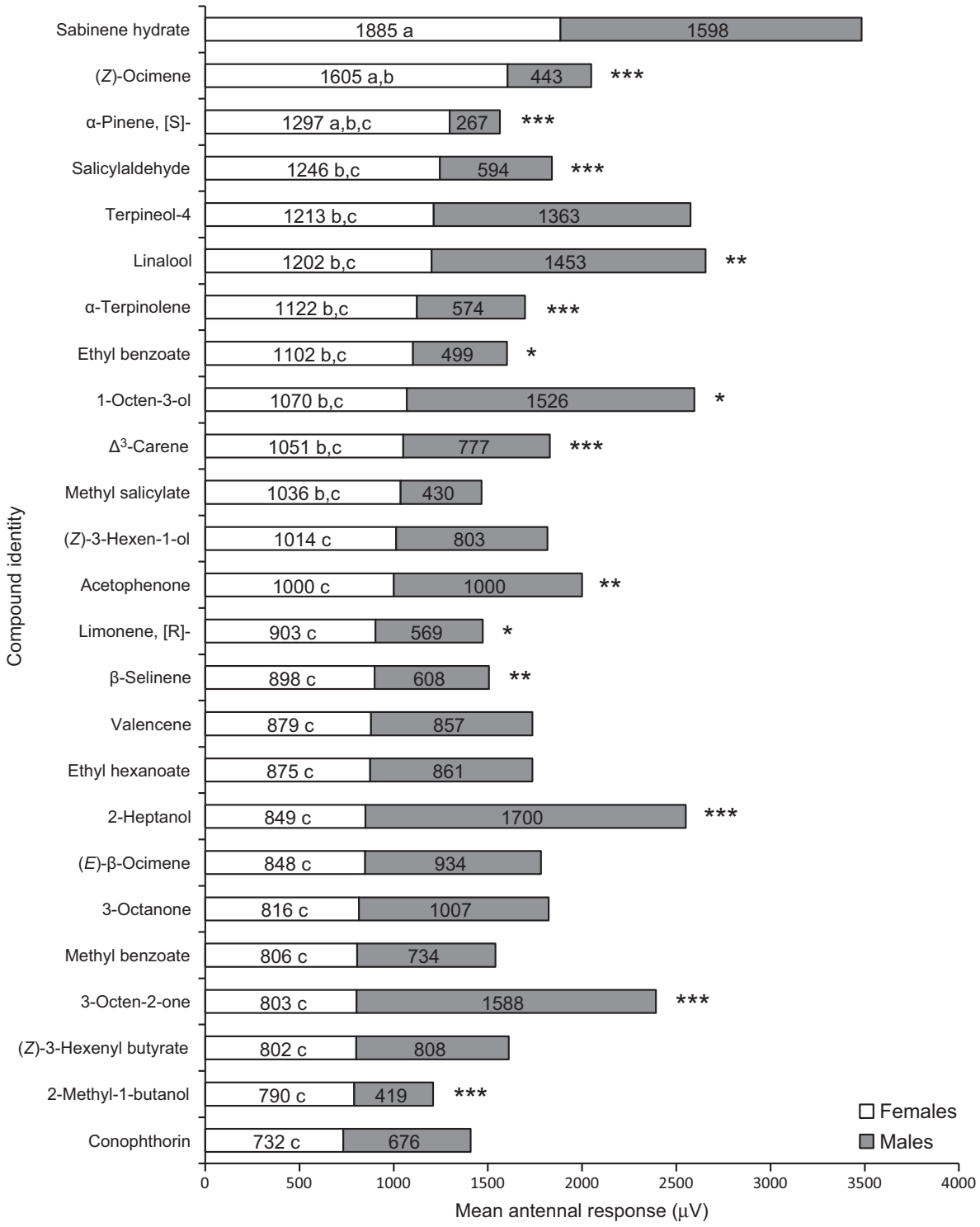

Figure 2 The top 25 compounds eliciting the highest electroantennographic responses (μV) from female *Amyelois transitella* and the associated male antennal response. Values are averages of n = 4–11 and are corrected to the antennal response to the standard acetophenone (1000 μV). Significant differences between female and male values are denoted by *0.01<P<0.05, **0.001<P<0.01, and ***P<0.001 (t-test). Different letters next to mean values for the female denote a significant difference between the responses to the individual volatiles (Tukey's test: P<0.05).

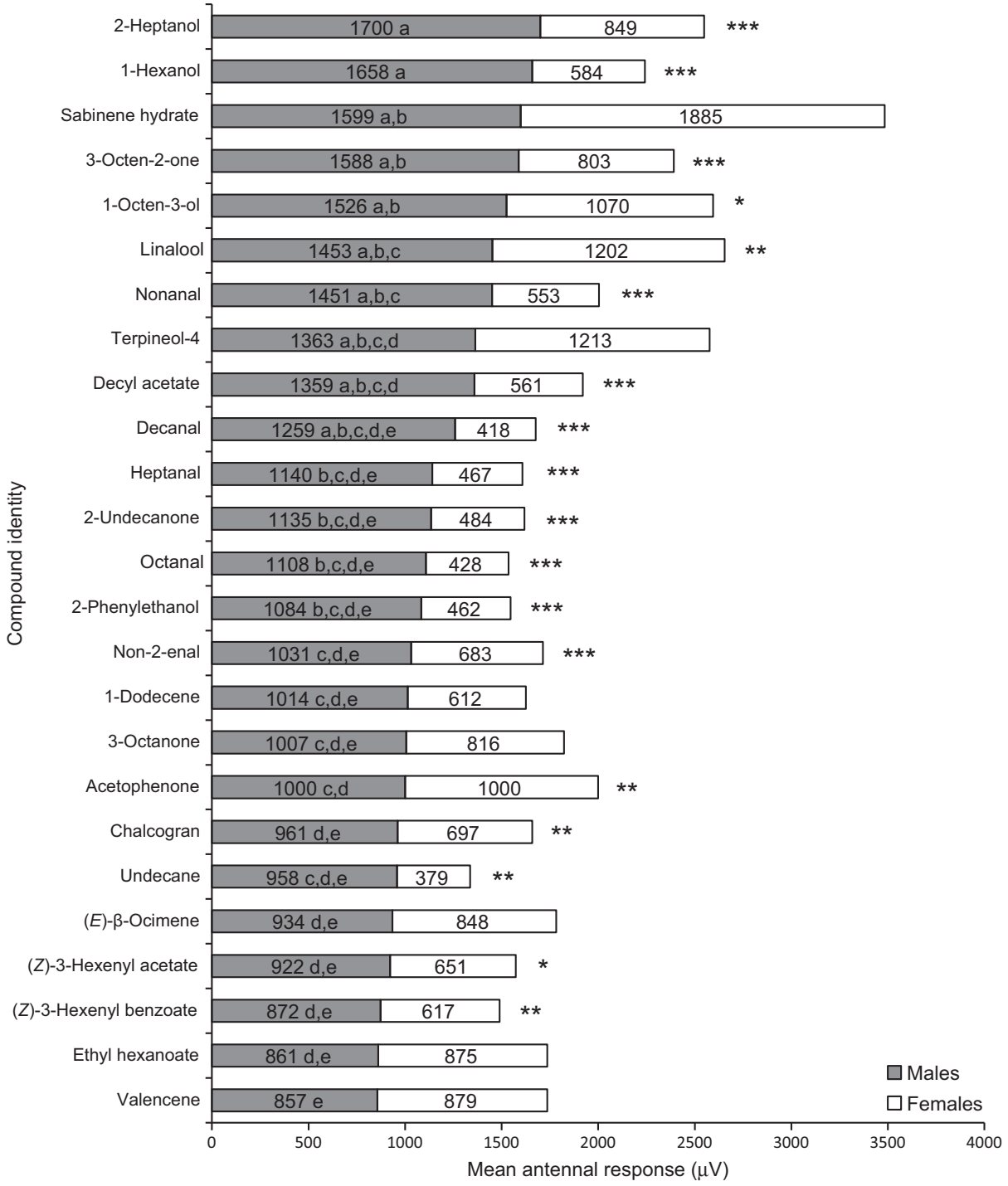

Figure 3 The top 25 compounds eliciting the highest electroantennographic responses (μV) from male *Amyelois transitella* and the associated female antennal response. Values are averages of n = 4–11 and are corrected to the antennal response to the standard acetophenone (1000 μV). Significant differences between female and male values are denoted by *0.01<P<0.05, **0.001<P<0.01, and ***P<0.001 (t-test). Different letters next to mean values for the male denote a significant difference between the responses to the individual volatiles (Tukey's test: P<0.05).

different amplitude responses from both female and male antennae.

Simple changes in a number of the benzenoids produced significant differences in antennal responses. For example, both male and female antennae responded well to acetophenone, yet when the methyl group is changed to hydrogen (to give benzaldehyde) the antennal response of both sexes drops significantly by more than half. Also of note was the significantly higher male antennal response to the alcohol 2-phenylethanol than to the corresponding aldehyde phenylacetaldehyde. Finally, there was no significant difference in response by both sexes to methyl salicylate and methyl benzoate, which only differ by a hydroxyl group at the ortho position. However, when the hydroxyl group of methyl salicylate is an amine, as in methyl anthranilate, the result was a 2–4× decrease in EAG amplitude for males and females, respectively.

The acyclic, monocyclic, and bicyclic subclasses of monoterpenes were among the top 10 volatiles eliciting the highest antennal responses for both sexes. For monocyclic monoterpenes, the relative locations of the alkenes had varying effects on the elicitation of antennal responses for both female and male navel orangeworm. For α- and γ-terpinene, the change from a conjugated and cyclic 1,3-alkene to the cyclic 1,4-alkene had no significant change in antennal response for males or females. Moving a cyclic alkene of α-terpinene to the exocyclic position to give α-terpinolene resulted in a significant difference in the response by the female antennae. Another movement of the exocyclic alkene to the terminal alkene of limonene numerically reduced the antennal responses of both sexes relative to α-terpinolene.

Hydration of the exocyclic alkene of α-terpinoline to give the tertiary alcohol terpineol-4 significantly increased responses for male, but not female antennae. Acetylation of the hydroxyl group of terpineol-4 to give α-terpinyl acetate significantly decreased antennal responses by 2.6× and 7× for females and males, respectively; thus suggesting an important role of the hydroxyl group in chemoreception.

In general, chirality of the tested monocyclic limonene enantiomers had no significant effect on responsiveness of the sexes. One exception was the chirality of α-pinene, which had significant effects with the (1S) enantiomer exceeding the (1R) enantiomer for female, but not male antennae. Repositioning of the cyclic alkene in (1S)-α-pinene to the terminal alkene in β-pinene significantly decreased the antennal response level for only females. The most dramatic structure-activity change for the monoterpenoids was the hydration of the terminal alkene in sabinene to give the tertiary alcohol, sabinene hydrate – the first and third ranked stimulating volatiles for female and male antennae, respectively; again suggesting the hydroxyl group as an important moiety for chemoreceptivity. Finally for the sesquiterpenes, the bicyclic volatiles valencene and β-selinene evoked significantly greater antennal responses for both sexes over the various acyclic, monocyclic, and tricyclic sesquiterpene volatiles tested.

The identification of several new compounds that elicited high EAG responses from navel orangeworm moth antennae, particularly terpenoids for females and fungal or fatty acid breakdown products for the male, provide new candidate semiochemicals for inclusion in ongoing studies. For instance, as pistachio orchards emit primarily monoterpenes (Roitman et al., 2011; Beck et al., in press) and Figure 2 showed that several monoterpenes elicited large antennal responses from female navel orangeworm, a logical start for the formulation of candidate blends should include volatiles from this class of compounds. The data and discussions provided support the primary goal of this study, which was to report these data to advance the development of monitoring lures and other potential semiochemical-based control tactics for the navel orangeworm moth. Furthermore, the chemoreception analysis and database presented provide a foundation for future electrophysiological and behavioural studies with *A. transitella*, and perhaps other Pyralidae.

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